THREE WAYS TO DIE SUDDENLY: DO THEY ALL REQUIRE CALCIUM CALMODULIN-DEPENDENT PROTEIN KINASE II?

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ABSTRACT

Sudden cardiac death occurs due to a limited number of pathological events. The heart can beat too fast or too slow to maintain adequate cardiac output or the heart can rupture. Here we survey recent evidence that excessive activation of calcium calmodulin-dependent protein kinase II by three core neurohumoral pathways or by oxidant stress can lead to sudden cardiac death due to sinus node dysfunction and bradycardia, ventricular tachycardia or fibrillation, and cardiac rupture.

INTRODUCTION

Sudden cardiac death is a major public health problem. Approximately 300,000 people die suddenly from cardiovascular diseases in the United States annually. Patients with structural heart disease and heart failure are at high risk for sudden cardiac death and the most common cause of structural heart disease leading to heart failure in the United States is myocardial infarction. A major portion of sudden deaths related to myocardial infarction occur acutely from ventricular fibrillation, but the incidence of other arrhythmias, including bradycardia and pulseless electrical activity, are on the rise (1). Bradycardia may be a more potent risk factor for sudden cardiac death than non-sustained ventricular tachycardia in heart failure patients (2). The advent of electrical defibrillation therapy and institution of cardiac intensive care units improved the acute survival of myocardial infarction patients primarily by reducing death from ventricular fibrillation. However, patients who survive this early phase of myocardial infarction are faced with additional chal-

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Potential Conflicts of Interest: Dr Anderson is the author of patents and patents pending claiming to treat various diseases by CaMKII inhibition. He is a cofounder of Allosteros Therapeutics, a biotech aiming to develop enzyme-based therapies.

lenges. Initial healing and scar formation involves a complex interplay between collagen deposition and breakdown where excessive activation of matrix metalloproteinases can promote myocardial instability and rupture (3), a rare but likely under-appreciated cause of sudden cardiac death (4). Surviving myocardium undergoes a variety of responses, including myocardial cell hypertrophy and reordering of cell membrane ion channel protein expression, a process called pro-arrhythmic electrical remodeling (5). Myocardial cell death and inflammation can increase fibrosis, contributing to inhomogeneous, pro-arrhythmic conduction (6). The cardiac chambers, particularly the left ventricle, may stretch and dilate (7). The net result of these processes is a maladaptive state of reduced mechanical function that contributes to the clinical syndrome of heart failure and defective cell membrane electrical excitability and defective tissue conduction that increase the probability of arrhythmias in patients who survive myocardial infarction.

Multiple systemic and cellular signals are affected in heart failure. However, work summarized elsewhere (8) from a growing number of investigators has indicated the multifunctional calcium (Ca^{2+}) and calmodulin-dependent protein kinase II (CaMKII) as a nodal signaling molecule with the potential to promote diverse disease pathways relevant to post-myocardial structural heart disease and arrhythmias. CaMKII is a serine threonine kinase that is abundant in myocardium. CaMKII exists as four distinct gene products (isoforms) with high inter-isoform homology. Each CaMKII monomer consists of three major domains: the C terminus association domain, the N terminus catalytic domain, and the interior regulatory domain (Figure 1). The catalytic domain contains the adenosine triphosphate binding pocket that is essential for catalyzing phosphorylation of consensus sequence serines and threonines. The regulatory domain contains a pseudosubstrate that constrains the catalytic domain under resting conditions to maintain most CaMKII in an inactive configuration. The C terminus association domain enables the CaMKII monomers to assemble into the holoenzyme. The CaMKII holoenzyme is built from a pair of stacked hexamers and the holoenzyme structure determines the molecular physiology of CaMKII (9). CaMKII is initially activated when intracellular Ca^{2+} increases (10), leading to increased levels of calcified calmodulin (Ca^{2+}/CaM). CaM is a ubiquitous Ca^{2+} sensing protein that binds Ca^{2+} via four EF hand domains. Ca^{2+}/CaM binds to a region of the CaMKII regulatory domain that distorts the pseudosubstrate-catalytic domain interactions, releasing the catalytic domain into an active configuration. There is a hypervariable region between the regulatory and association domains that is expressed as a number of

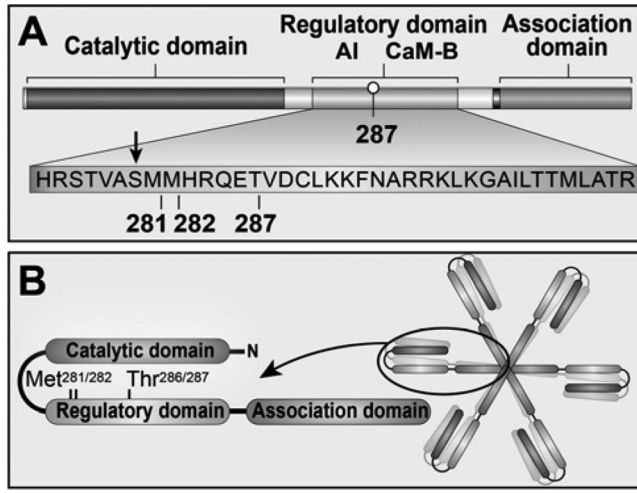

FIG. 1. Domain structure of CaMKII. **(A)** A schematic depiction of a CaMKII monomer. The regulatory domain consists of an autoinhibitory region (AI) and a calmodulin binding region (CAM-B). Key amino acid residues in the AI are shown. The *downward arrow* indicates a site for glucose modification, the paired methionines are sites for oxidation and threonine 287 is a site for autophosphorylation (see text). **(B)** A schematic depiction of a CaMKII hexamer. Each CaMKII holoenzyme consists of a pair of hexamers. Here CaMKII is in an inactive conformation due to constraint of the catalytic domain by the AI.

splice variants. The longer hypervariable splice variants are activated by lower amounts of Ca^{2+}/CaM compared to CaMKII with shorter splice variants (9). If elevation of intracellular Ca^{2+} is brief (a few milliseconds) and reactive oxygen species (ROS) are not abundant, activated CaMKII reverts to an inactive conformation upon Ca^{2+}/CaM unbinding. However, if intracellular Ca^{2+} elevations are sustained or occur under conditions of high ROS, CaMKII transitions to a constitutively active, Ca^{2+}/CaM-independent conformation, by one of three processes: autophosphorylation (11), oxidation (12), or O-linked N-acetylglucosamine (O-GlcNAc) (13). Autophosphorylation of a regulatory domain threonine 286/287 (the numbering varies slightly between isoforms) enhances the avidity of Ca^{2+}/CaM binding (so-called CaM "trapping") but also sustains CaMKII activity after Ca2+/CaM unbinding (14). Our group identified a pathway where oxidation of a pair of regulatory domain methionines (281/282) locked CaMKII into a persistently active configuration (12). The first oxidation step (to a sulfoxide) is reversible. Reduction of oxidized CaMKII (ox-CaMKII) is catalyzed by methionine sulfoxide reductase A (MsrA) (12). Very recently, a regulatory domain serine (280) has been identified as a site for

covalent modification by O-GlcNAc, a process that is favored under hyperglycemic conditions and leads to Ca^{2+}/CaM-independent activity (13). The ability of CaMKII to transition between a Ca^{2+}/CaM regulated activity state and a Ca^{2+}/CaM autonomous enzyme has important implications in cardiovascular and pulmonary disease; in general, disease processes are promoted by constitutively active forms of CaMKII.

CaMKII is abundant in myocardial tissue where it appears to enhance performance to physiological responses to stress, such as the fight or flight response mediated increases in heart rate (15). However, excessive CaMKII activation that is present in myocardium challenged by pathological stressors is now thought to contribute to diseases by promoting diverse mechanisms (Figure 2). This manuscript will review published data from our laboratory to promulgate a novel hypothesis that excessive myocardial CaMKII activity contributes to diverse causes of sudden cardiac death.

RESULTS

CaMKII AND TACHYARRHYTHMIAS

Patients who are at highest risk for sudden cardiac death due to rapid (tachy) arrhythmia (ventricular tachycardia or ventricular fibrillation) are those in the throes of a myocardial infarction or with significant structural heart disease, including severe myocardial hypertrophy and/or heart failure. Certain genetic diseases of ion channel proteins and proteins involved in targeting of ion channels to specific subcellular domains also increase the probability of sudden death by tachyarrhythmias. CaMKII expression and/or activity are increased during ischemic injury (16) and in myocardium from patients and animal models with structural heart disease and heart failure (17). CaMKII activity is also implicated as a pro-arrhythmic signal in genetic arrhythmia syndromes, including the long QT syndromes (18–21) and catecholaminergic ventricular tachycardia (22, 23).

CaMKII appears to contribute to arrhythmias by two general mechanisms: pro-arrhythmic electrical and substrate remodeling. CaMKII catalyzes phosphorylation of all (to my knowledge) voltage-gated ion channels in myocardium. CaMKII phosphorylation of the ryanodine receptor, the largest ion channel and principle egress pathway for Ca^{2+} in the sarcoplasmic reticulum lumen to enter the cytoplasm (24), leads to Ca^{2+} "leak" and activation of the Na^{+}/Ca^{2+} exchanger (25, 26). The net effect of CaMKII activity on myocardial cell membrane conductance pathways is to enhance cell membrane excitability (Figure 3).

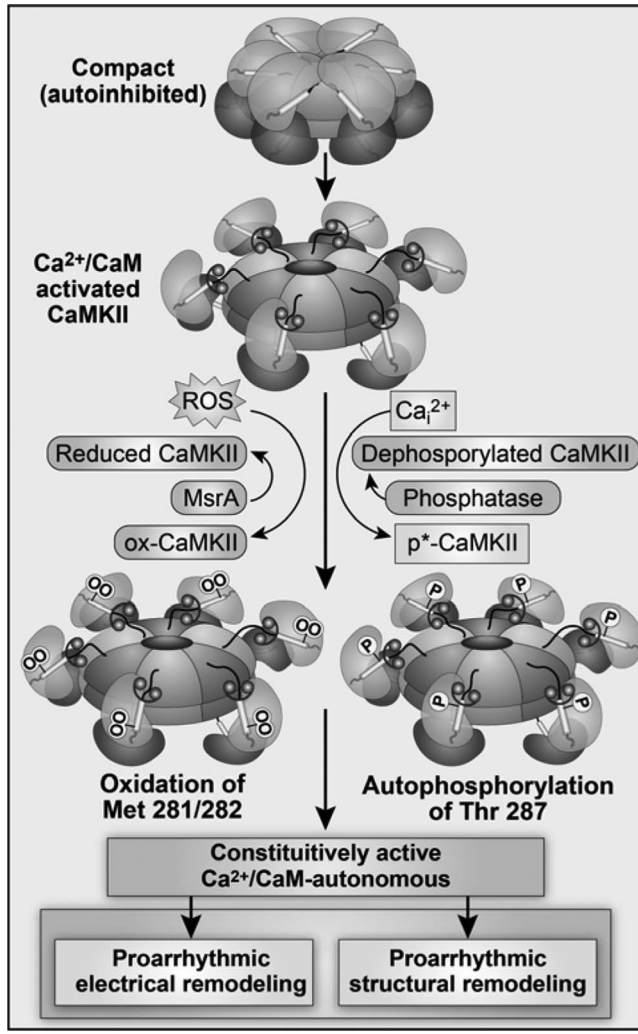

FIG. 2. Relationship between constitutively activated CaMKII and pro-arrhythmic electrical and structural remodeling. Top figure shows the compact inactivated conformation of CaMKII. The lower figure shows calcium and calmodulin (Ca^{2+}/CaM) activates CaMKII by inducing an extended conformation. The lowermost figures show ROS or autophosphorylation (see text) induce a Ca^{2+}/CaM autonomous extended CaMKII conformation that promotes pro-arrhythmic electrical and structural remodeling in myocardium. (Abbreviation: MsrA, methionine sulfoxide reductase A.)

Enhanced cell membrane excitability manifests as depolarizing oscillations in cell membrane potential called afterdepolarizations. Afterdepolarizations that reach a critical threshold of membrane depolar-

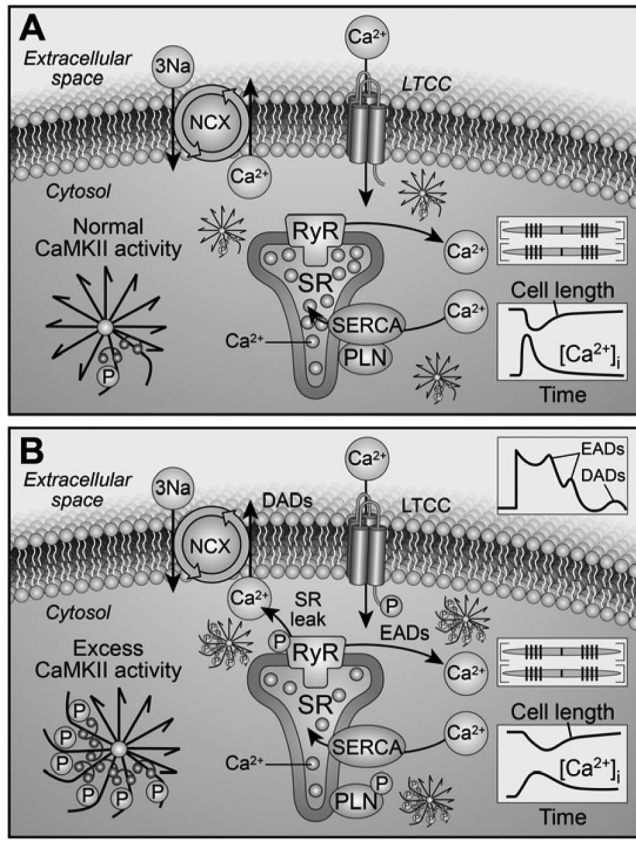

FIG. 3. Physiological and pathophysiological roles of CaMKII in cardiac excitation-contraction coupling. **(A)** Physiological CaMKII activation coordinates myocardial Ca^{2+} entry through L-type Ca^{2+} channels (LTCC) for regenerative release of sarcoplasmic reticulum (SR) Ca^{2+} through ryanodine receptors (RyR). **(B)** Excessive, pathophysiological, CaMKII activation contributes to arrhythmias by LTCC phosphorylation and increased channel opening probability, SR Ca^{2+} leak, promotion of inward Na^{+}/Ca^{2+} exchanger (NCX) current, and arrhythmia-triggering cell membrane potential oscillations known as delayed afterdepolarizations (DADs).

ization trigger aberrant action potentials. These triggered beats can occur rapidly, leading to ventricular tachycardia, or occur at a vulnerable time inducing ventricular fibrillation (eg, R on T). CaMKII also contributes to a pro-arrhythmic substrate by promoting myocardial hypertrophy, myocardial death, fibrosis, and inflammation (27, 28). Collectively, these processes lead to pro-arrhythmic inhomogeneity in conduction that favors electrical "re-entry," a regenerative process that sustains ventricular tachycardia and fibrillation (Figure 4).

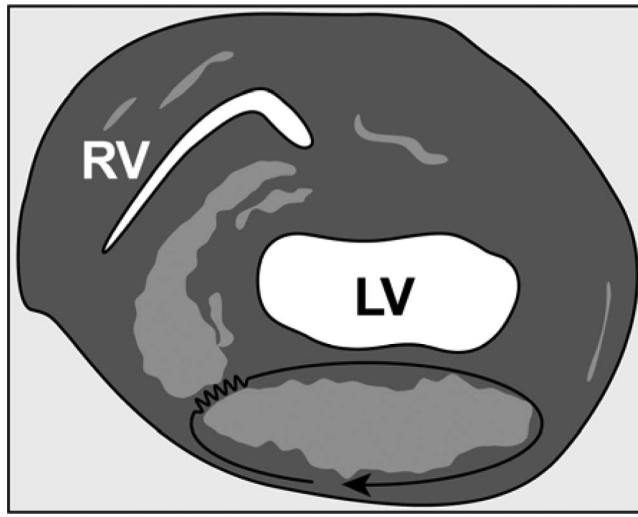

FIG. 4. Electrical re-entry pathway in heart is a representation of pro-arrhythmic structural remodeling due to scar formation. The *arrow* shows the reentry pathway and the *undulating line* indicates a region of slowed conduction. (Abbreviations: RV, right ventricle; LV, left ventricle.)

The potential role of CaMKII has not yet been tested in humans because clinical drugs are lacking. However, there is now a large body of evidence in mice and in large animal models, using pharmacological and genetic approaches, showing that CaMKII inhibition can reduce or prevent ventricular tachycardia and ventricular fibrillation (5).

CaMKII AND BRADYARRHYTHMIAS

Bradyarrhythmias can be due to defects in sinoatrial nodal (SAN) pacemaker cells, scarring of tissue surrounding the SAN that interrupts (blocks) conduction to surrounding atria or to faulty conduction in the specialized atrial nodal or bundle branch conduction tissue. Physiological SAN pacing transpires by two basic mechanisms: inward currents that lead to cell membrane depolarization such as HCN4 that are independent of intracellular Ca^{2+} (29), and inward current due to the Na^{+}/Ca^{2+} exchanger that is activated by release (leak) of Ca^{2+} from the sarcoplasmic reticulum (30). The latter mechanism is very similar to afterdepolarizations that are an initiating cause of tachyarrhythmias (discussed above). SAN cells pace the heart by injecting current that is sufficient for high fidelity capture of the surrounding atrial myocardium. We discovered that CaMKII activity enables physiological fight or flight heart rate increases by phosphorylating the SAN

ryanodine receptors, enhancing sarcoplasmic reticulum Ca^{2+} release in late diastole that activates inward Na^{+}/Ca^{2+} exchanger current (15). In fact, CaMKII is required for approximately half of the dynamic range of heart rate increases in response to isoproterenol. Thus, CaMKII appears to be an important constituent of SAN cells because of its role in increasing heart rates in response to physiological stress.

Sinus node dysfunction, loss of heart rate variability, and bradycardia are risk factors for increased mortality in common cardiac conditions, including recovery from myocardial infarction, heart failure, and diabetes. Based on the role of CaMKII in SAN physiology and the general concept that a core feature of myocardial disease is increased ROS and disturbed intracellular Ca^{2+} homeostasis, we asked if excessive CaMKII could contribute to SAN dysfunction and/or bradycardia. Because angiotensin II (Ang II) levels are elevated in heart failure patients, we infused mice with Ang II over 3 weeks to achieve plasma levels similar to those measured in patients with heart failure. The Ang II–infused mice developed profound bradycardia that was due to excessive ox-CaMKII–mediated SAN cell apoptosis and atrial fibrosis (31). We used a mathematical model to identify a critical threshold for SAN cell death that led to a loss of high fidelity atrial capture and bradycardia (31). A talented cardiovascular medicine fellow in our laboratory, Dr Paari Swaminathan, adapted a gene painting technique to deliver a CaMKII inhibitor encoding adenovirus directly to the SAN. Gene painted mice, mice with "global" myocardial CaMKII inhibition and mice lacking *p47*, a necessary constituent of Ang II responsive NADPH oxidases, were resistant to Ang II–induced increases in ox-CaMKII, SAN cell death, and SAN dysfunction. To determine if our findings in mice were potentially relevant to mechanisms active in patients with SAN dysfunction, we obtained right atrial tissue from heart failure patients. Some of these patients required a permanent pacemaker for SAN dysfunction and others had no pacemaker and no documented SAN dysfunction. We found that right atrial tissue, near the SAN region, from heart failure patients with SAN dysfunction had significantly more ox-CaMKII than in tissue samples from patients with heart failure but no evidence of SAN dysfunction. We interpreted our findings to suggest that Ang II–induced ROS increases could increase SAN ox-CaMKII, leading to excessive SAN cell death, sinus node dysfunction (SND), and bradycardia.

Most diabetic patients die from cardiovascular disease and diabetic patients are approximately twice as likely to die as non-diabetic patients after myocardial infarction and this mortality excess persists after adjusting for common clinical risk factors, such as extent of

coronary disease and left ventricular ejection fraction (32). Furthermore, diabetes is marked by increased myocardial ROS, suggesting to us that ox-CaMKII could contribute to cardiovascular disease in diabetes. Another talented cardiovascular medicine fellow, Dr Min Luo, rotated in our laboratory with the goal of testing if CaMKII could contribute to diabetic cardiomyopathy. She first measured ROS and ox-CaMKII in heart samples from diabetic and non-diabetic patients after myocardial infarction and found that diabetic myocardium had significantly increased ROS and ox-CaMKII compared to non-diabetic controls. She used a model of severe type I diabetes in mice by injection of the pancreatic β cell toxin streptozocin (STZ). Similar to the clinical scenario, diabetic mice had approximately twice the mortality in the first week after myocardial infarction surgery compared to non-diabetic control mice after myocardial infarction. To our surprise, diabetic and non-diabetic mice had similar left ventricular function after myocardial infarction surgery, suggesting that excess diabetes-attributable mortality was not related to heart failure. We repeated this study in mice implanted with electrocardiogram telemeters to determine if arrhythmias could account for excess mortality in diabetic mice after myocardial infarction. We found that diabetic mice had markedly reduced heart rate variability, a validated risk factor for sudden death (33, 34), and died with severe bradycardia (35). Similar to Ang II–treated mice (31), diabetic mice had high levels of SAN cell ROS, ox-CaMKII, and apoptosis, suggesting diabetes increased ox-CaMKII causing bradycardia. To test if ox-CaMKII was a critical signal promoting SAN dysfunction and increased mortality, we repeated the STZ/myocardial infarction studies in mice developed by our group where methionines 281/282 in the predominant myocardial CaMKII isoform (CaMKIIδ) underwent knockin replacement with valines (MM281/282VV). Remarkably, the MM281/282VV mice were resistant to the diabetes-attributable mortality after myocardial infarction. In contrast to our studies in Ang II–infused mice, we found that the $p47^{-/-}$ mice were not protected from increased death and that increased myocardial ROS in diabetes arose from a mitochondrial source. Based on this finding, we infused mice with mitoTEMPOL, a mitochondrial targeted antioxidant (36). Similar to the MM281/282VV mice, mitoTEMPOL-infused wild type mice were resistant to diabetes-attributable mortality after myocardial infarction. We interpreted these data to show that diabetic patients had more myocardial ox-CaMKII than non-diabetic patients and to suggest that diabetes increased mortality after myocardial infarction, at least in part, by ox-CaMKII–triggered SAN dysfunction and bradycardia.

CaMKII AND CARDIAC RUPTURE AFTER MYOCARDIAL INFARCTION

Our laboratory identified CaMKII as a pathological downstream signal for cardiotoxic actions of β-adrenergic receptor agonists (37) and Ang II (12). Based on these findings, we wondered if aldosterone, a component of the renin-angiotensin-aldosterone signaling pathway, was also an upstream agonist for CaMKII activation in myocardium. We found that aldosterone increased myocardial ROS by a pathway that required mineralocorticoid receptors and nicotinamide adenine dinucleotide phosphate oxidase. The increases in ROS occurred within minutes of adding aldosterone to cultured heart cells, and so were not likely to involve genomic or transcriptional signaling directly. The increased ROS lead to commensurate increases in ox-CaMKII. Because the clinical benefits of mineralocorticoid antagonists were mainly found in patients with heart failure due to myocardial infarction, we chronically infused mice with aldosterone starting at the time of myocardial infarction surgery. The aldosterone infusion resulted in aldosterone plasma concentrations at the lower end of the range from aldosterone measurements in heart failure patients in the RALES trial (38). Although we hypothesized that elevated aldosterone levels would increase heart failure, we found that mice infused with aldosterone or saline had similarly decreased left ventricular function and dilation after myocardial infarction surgery. Nevertheless, aldosterone infusion significantly increased the 7-day post-myocardial infarction mortality and that this excess mortality was due to myocardial rupture. We hypothesized that the increased myocardial rupture was due to excessive activity of a collagenase, matrix metalloproteinase 9 (MMP9), because MMP9 was an established signal that increased after myocardial infarction and that was associated with left ventricular rupture in mice (3). To test if MMP9 was relevant to patients, we measured MMP9 expression from hearts of patients who died in the first week after myocardial infarction in the presence or absence of left ventricular rupture. We found that ruptured myocardium had significantly higher levels of MMP9 compared to non-ruptured myocardium. Surprisingly, excess MMP9 was also present in myocardial cells, suggesting that pathologically stressed myocardium was capable of expressing MMP9 (39).

The increase in MMP9 expression caught our attention because we had identified *MMP9* as a signal that was positively regulated by myocardial infarction in wild-type mice but not in mice with myocardial-delimited, transgenic expression of AC3-I, a CaMKII inhibitory peptide modeled after the CaMKII autoinhibitory region of the regulatory domain (27). These array studies suggested that pathologically

stressed myocardium was expressing MMP9 by a CaMKII-dependent process. We used a cohort of genetically modified mice to delineate a pathway where aldosterone and myocardial infarction increased ox-CaMKII, increased CaMKII activity, and activated a myocyte enhancer factor 2 promoter on the *MMP9* gene in myocardium. We found that myocardial sourced MMP9 was critical for the increased risk of cardiac rupture after myocardial infarction in aldosterone-infused mice. Mice with myocardial delimited expression of AC3-I or myocardial delimited MsrA overexpression were highly resistant to left ventricular rupture in the setting of myocardial infarction and aldosterone infusion. In contrast, mice lacking MsrA ($Msra^{-/-}$) showed increased susceptibility to cardiac rupture under these conditions (39).

DISCUSSION

Our laboratory has developed evidence that CaMKII is a pleotropic signal activated by upstream signals common to many adult diseases, defective cellular Ca^{2+} homeostasis, and increased ROS. Here I have assembled evidence to support a hypothesis that hyperactivation of CaMKII is a central event in diverse causes of sudden cardiac death, a clinical phenotype without adequate treatments. Although our group and our collaborators have assembled evidence from human tissues that support the contention that our hypothesis is clinically relevant, in my opinion, the true test of this hypothesis requires development of CaMKII inhibitory drugs that can be deployed in patients. Although the time frame for developing such drugs is uncertain, several groups and pharmaceutical companies have CaMKII inhibitory drug discovery programs. Thus, I am hopeful that the potential translational value for CaMKII inhibition for cardiovascular and pulmonary diseases will be understood in my lifetime.

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